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ITIH5, a novel member of the inter-α-trypsin inhibitor heavy chain family is downregulated in breast cancer

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Abstract

The inter-α-trypsin inhibitor (ITI) family constitutes a group of proteins built up from one light chain and a variable set of heavy chains. Originally identified as plasma protease inhibitors, recent data indicate that ITI plays a role in extracellular matrix (ECM) stabilization and in prevention of tumor metastasis. Here we describe cloning as well as phylogenetic and expression analysis of a novel member of the heavy chain gene family, *ITIH5*. ITIH5 contains the two domains conserved in all known ITIHs, the vault protein inter-alpha-trypsin (VIT) domain and a von Willebrand type A (vWA) domain. However, ITIH5 diverged early from a common ancestor of the other subfamilies. We found strong downregulation of *ITIH5* expression in breast tumors by real-time PCR and RNA in situ hybridization. While normal breast epithelial cells clearly express *ITIH5*, expression is consistantly lost or strongly downregulated in invasive ductal carcinoma. *ITIH5* mRNA was neither detectable in cancerous nor benign breast cell lines. We propose that loss of *ITIH5* expression may be involved in breast cancer development.

Keywords: Inter-α-trypsin inhibitor; ITI heavy chain; Breast cancer; Tumor suppressor gene

1. Introduction

The inter-α-trypsin inhibitor (ITI) family is composed of protease inhibitors that are assembled from two precursor proteins: a light chain and different homologous heavy chains. To date, the family of heavy chains (*ITIHs*) consists of four members, *ITIH1*, *ITIH2*, *ITIH3* and *ITIH4*, encoded by four different genes located on two different chromosomes [1]. *ITIH1*, *ITIH3* and *ITIH4* have been mapped to chromosome 3p2.11-12, whereas the *ITIH2* gene is located on chromosome 10p14-15 [2]. All heavy chains, except ITIH4, are synthesized as polypeptide precursors primarily in the liver and undergo extensive posttranslational processing. ITIH1, −2, and −3 contain a conserved cleavage site and are able to form covalent bonds to bikunin

Abbreviations: ITI, inter-α-trypsin inhibitor; ITIH, (ITH), inter-α-trypsin inhibitor heavy chain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; IDC, invasive ductal carcinoma.

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(ITI light chain) via glycosaminoglycan [3]. The conserved sequence for C-terminal cleavage is absent in ITIH4, thus preventing a bond with bikunin [4].

A potential role for ITI proteins in tumor invasion has been postulated on the basis of their protease inhibitor function, mediated by bikunin [5], and their capacity to bind to the extracellular matrix (ECM) component hyaluronan, mediated by the heavy chains [6–8]. Recently, it was shown that overexpression of ITI family chains leads to inhibition of tumor development and/or metastatic spreading. Bikunin overexpression causes a dramatic decrease of primary tumor weight and metastasis number in lung cancer mouse models, whereas *ITIH1* and *ITIH3* overexpression only induces a decrease of metastasis number. In vitro experiments showed that *ITIH1* and *ITIH3* expression increased cell attachment [9]. Here we describe cloning and expression analysis of a novel member of the *ITIH* gene family named *ITIH5*. We demonstrate that *ITIH5* transcript is clearly downregulated in breast tumors. Therefore, we propose that loss of *ITIH5* may play a role in breast cancer development.

2. Materials and methods

2.1. Tissues

Frozen human breast tumors and normal tissues were retrieved from the Department of Gynecology, Friedrich Schiller University Jena, Germany. These samples were snap-frozen and stored in liquid nitrogen.

2.2. Cell lines and culture conditions

The following breast carcinoma cell lines were maintained in RPMI 1640 media: MCF7, ZR-75.1, T47D, and BT-474. MCF10A cells, MDA-MB-468 and MDA-MB-231 were maintained in DMEM. SK-BR-3 cells were cultured in McCoy's 5a media. MDCB media 105 and 199 (1:2) were used to maintain the ovary adenocarcinoma cell lines TOV112D and TOV21G. All culture media were supplemented with 10% fetal bovine serum and 2 mM L-glutamine.

2.3. Multiple alignment and phylogenetic tree construction

We scanned several databases to identify known protein sequences of the *ITIH* gene family. Multiple alignments were carried out with CLUSTALX version 1.8.1 [10] using the BLOSUM62 substitution matrix. The neighbour-joining algorithm [11] was used to build the phylogenetic tree after gaps were removed from the alignments. The significance of each node was determined by calculation of 1000 bootstrap replicates. The MEGA package was utilized to build and visualize the tree [12].

2.4. Cloning of the ITIH5 cDNA

First strand cDNA was synthesized by reverse transcription (RT) of poly(A)$^+$ RNA from normal breast tissue using Superscript™ II reverse transcriptase (Invitrogen, Karlsruhe, Germany) and oligo(dT) primers. *ITIH5* cDNA was amplified by PCR using proof-reading Platinum Pfx DNA polymerase (Invitrogen, Karlsruhe, Germany) and the following primers:

forward 5′-CGCGGGATCCATGCTCCTGCTGC-TGGGG-3′ and reverse 5′-ATAGTTTAGCGG-CCGCTCACTGTCCTTCATGCAC-3′ (primers contain start and stop codons). The PCR consisted of 30 cycles of 15 s at 94 °C, 30 s at 55 °C, and 3 min at 68 °C. PCR was preceded by 2 min at 95 °C and followed by 7 min at 68 °C. The PCR product was analyzed by agarose gel electrophoresis, and the band of interest was purified by High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The purified *ITIH5* fragment was cloned into pBK-CMVΔ vector (Stratagene, Amsterdam, The Netherlands) and sequenced. Genbank Acc. No. is AY238437.

2.5. Northern blot and tumor expression array

Multiple Tissue Northern Blot (MTN) and Human Matched Tumor/Normal Expression Array (Clontech, Palo Alto, USA) were hybridized with 25 ng of a ^{32}P-labeled fragment of 3′-*ITIH5* cDNA clone (Gen Bank Acc. No: AA134750) and a β-actin cDNA probe (Clontech) was used as a control.

2.6. Real-time PCR

The real-time PCR was performed using the Gen Amp® 5700 sequence detection system (PE Applied Biosystems, Weiterstadt, Germany). We used the following intron-spanning primers: forward: 5′-ACGCACACCCTCAAGATCCT-3′; reverse: 5′-ATGCCAATGGTGAAGATGCA-3′ and FAM (5′ end)/TAMRA (3′ end)-labeled specific probe 5′-CAACACCCGAGAGGCCGCCC-3′.

Each PCR reaction was performed in a 25 μl volume that included 12.5 μl 2 × TaqMan Universal PCR-Mastermix (PE Applied Biosystems, Weiterstadt, Germany), 1 ng of cDNA template, 300 nM of each primer, and 100 nM of the specific probe. The relative RNA quantification was performed with comparative C_T method as described by the manufacturer. The housekeeping gene GAPDH was used as reference

(GAPDH primers:
forward: 5′-GAAGGTGAAGGTCGGAGTC-3′;
reverse: 5′-GAAGATGGTGATGGGATTTC-3′;
probe: 5′-CAAGCTTCCCGTTCTCAGCC-3′).

2.7. Radioisotopic RNA in situ hybridization

Radioactively labeled partially hydrolysed riboprobes were generated from a clone containing full-length *ITIH5* cDNA. RNA in situ hybridization was performed as previously described [13]; instead of ^{32}P we used ^{33}P rUTP. In brief, serial cryo-sections (5 μm) were mounted on 3-aminopropyl-triethoxysilane-coated slides, fixed in 4% paraformaldehyde in 2 × SSPE, digested with proteinase K (0.5 μg/ml), and pre-hybridized at 60 °C for 2–4 h. Hybridization was performed overnight at 60 °C in 50% formamide, 2 × SSPE, 10% dextran sulfate, 10 mM Tris–HCl pH 7,5; 1 × Denhardt's solution, 500 μg/ml tRNA, 100 μg/ml herring sperm DNA, 0.1% SDS, and 10^5 cpm radioactive probe/μl. After hybridization, slides were washed once in 50% formamide, 2 × SSPE, 0.1% SDS for 30 min at 50 °C, treated with RNaseA (50 μg/ml in 2 × SSC, 0.1% SDS), and washed again in 50% formamide, 0.5 × SSPE, 0.1% SDS for 30 min at 37 °C. Slides were dehydrated in alcohol, dried, dipped in film emulsion (Kodak NTB 2, diluted 1:1 with 600 mM ammonium acetate) and exposed for 7–21 days at 4 °C.

3. Results and discussion

3.1. Cloning and characterization of ITIH5

Applying an EST-based bioinformatics approach previously described in Schmitt et al. [14] we identified *ITIH5* as a novel gene differentially expressed in breast tumor tissue. Subsequently, an *ITIH5* cDNA clone containing the complete coding sequence (2828 bp) was isolated from mammary gland tissue. Sequencing analysis showed that our sequence is highly similar to a sequence in public databases (Acc. No. NM_030569). Compared to this sequence our *ITIH5* cDNA from breast tissue (Acc. No. AY238437) contains two nucleotide exchanges leading to amino acid substitutions in the derived protein sequence at positions 188 (Asp instead of Gly) and 337 (Ser instead of Pro) respectively. The genomic organization of human *ITIH5* gene was determined by comparing cDNA sequence to the human genome database. The *ITIH5* gene spans 82.7 kb and is composed of 14 exons (Fig. 1). It is localized on chromosome 10p15 separated by 36.4 kb from the *ITIH2* gene. A similar tandem organization has been described for two other family members, *ITIH1* and *-3*, on chromosome 3p2.11-12 [1]. However, *ITIH2* and *ITIH5* are transcribed in opposite directions and the large distance between both genes argues against a common regulation on the transcriptional level.

Fig. 1 further illustrates the domain structure of ITIH5 which constitutes a polypeptide of 942-amino acid residues. It contains a vault protein inter-alpha-trypsin (VIT) domain (residues 44–161) and a von Willebrand type-A (vWA) domain (residues 295–478). These two domains are conserved in all known ITIHs. The vWA domain has a binding capacity for integrins, collagens, proteoglycans and heparin [15]. In ITIHs, the vWA domain is typically associated with an N-terminal VIT domain. The VIT domain is less widespread than the vWA domain and is not genetically mobile. Therefore, it can be regarded as the characteristic domain of the ITIH family. In contrast to the vWA domain, the structure of the VIT domain has not been solved yet. ITIH5 is further characterized by a signal peptide (residues 1–18) at the N-terminus and a conserved cleavage site (DPHFVV) in the C-terminal segment that is trimmed

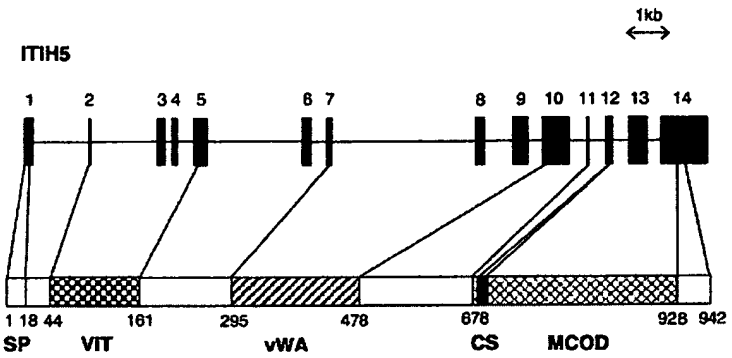


Fig. 1. Genomic organization and domain structure of the *ITIH5* gene. The 14 exons of *ITIH5* are represented by black rectangles and are numbered 1–14. The line between the exons indicates introns which are shown at a reduced scale (1:10). Overview of Symbols: grey area, signal peptide (SP); check-board area, vault protein inter-alpha-trypsin domain (VIT); hatched area, von Willebrand type A domain (vWA); crossed hatched area, multicopper oxidase domain (MCOD); black box, conserved cleavage site (CS = DPHFVV).

off during chain assembly. The role of the trimmed C-terminal segment is yet unknown. However, this domain does contain a multicopper oxidase domain (residues 678–928) that is able to bind copper [16]. The multicopper oxidase domain is present in ITIH1 and −3 but absent in ITIH2 and −4. From the characteristic domain structure of ITIH5 which is similar to known ITIHs we concluded that it constitutes a novel member of the ITIH superfamily.

To further analyze the similarity to other ITIHs, we collected sequence information from all known mammalian ITIH proteins and performed a phylogenetic analysis. We predicted and excised all VIT and vWA domain sequences of the ITIH proteins. For these conserved sequence regions we were able to yield alignments of high quality. The independent VIT and vWA alignments were fused into one large alignment (Fig. 2). The VIT domain is approximately 135 amino acids long. In the N-terminal part of the VIT domain, we observed a pattern of hydrophobic residues, interrupted by a conserved arginine. The C-terminus is best characterised by its conserved aromatic residues. In the central part, an acidic amino acid resides between two basic residues. We hypothesize that the hydrophobic N-terminus and the aromatic pattern of amino acids are essential for the structural integrity of the domain. The alternating charges most likely contribute to the functionality of the ITIH heavy chains.

The combined alignment served as the basis for the construction of a phylogenetic tree (Fig. 3). To get an estimate for the reliability of the topology we performed 1000 bootstrap replicates. The tree revealed that the novel ITIH5 proteins form a distinct clade which is separated from the other ITIH subfamilies by 100% bootstrap support. All the ITIH subfamilies 1–5 are separated clearly from each other. It seems that the ITIH5 subfamily has diverged early from a common ancestor of the other four subfamilies. By BLAST searches, we collected evidence for the existence of ITIH like proteins in fishes and even in *Ciona intestinalis*. Future data on the complete genomes of these and other chordates will be helpful in the reconstruction of the evolutionary origin of each ITIH subfamily and could provide important links to their functional divergence.

3.2. Expression of ITIH5 in normal tissues and breast tumors

Expression of *ITIH5* mRNA in normal human tissues was examined by Northern blot analysis. A transcript of 3.6 kb in size could be detected in several tissues (Fig. 4A). Although this transcript was only weakly expressed in most tissues, it was abundantly expressed in placenta. Real-time PCR analysis (Fig. 4C) confirmed the predominant expression of *ITIH5* in placenta. Less abundant expression was

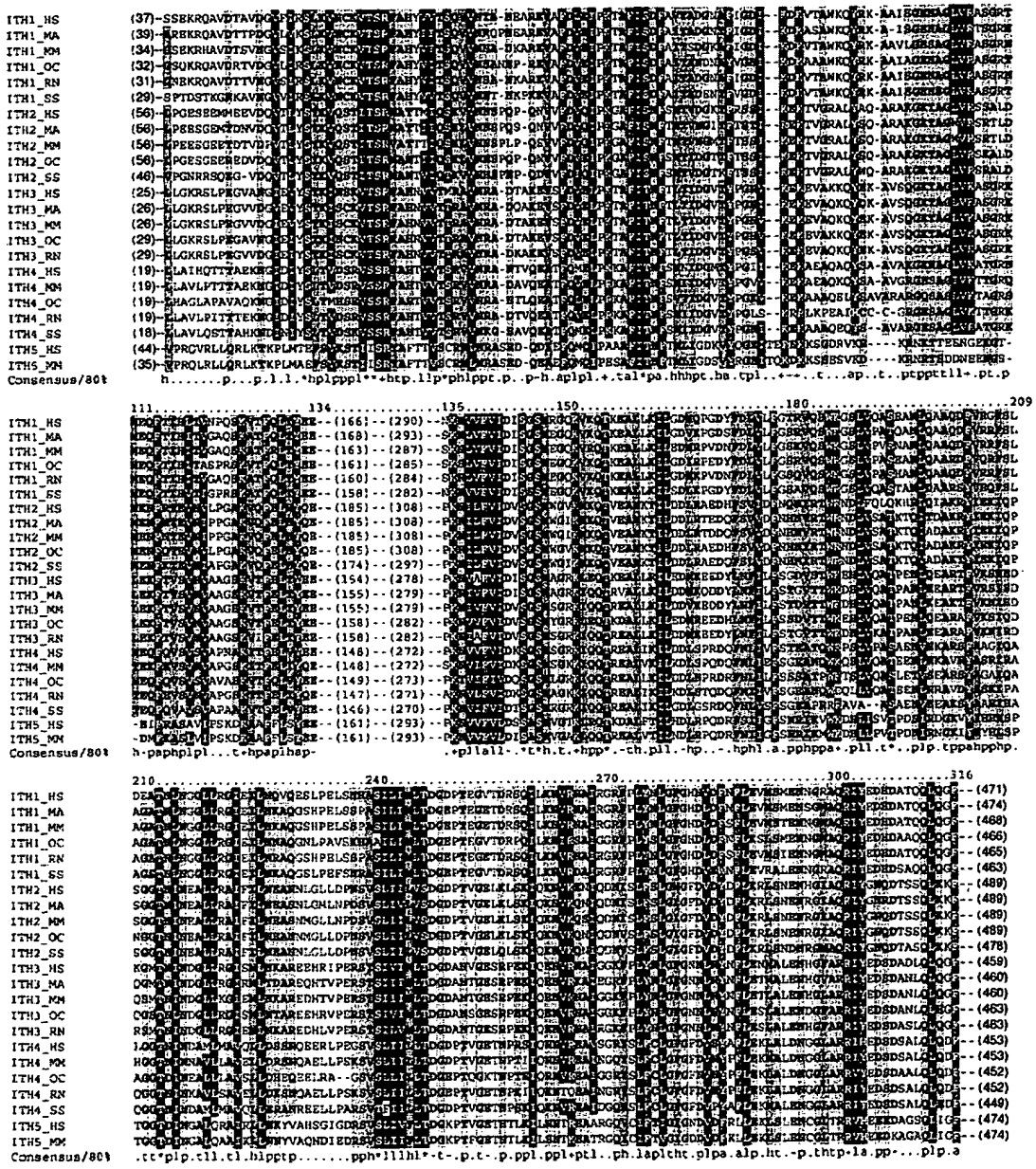


Fig. 2. Alignment of members of the ITIH protein family. The consensus string is given at the bottom of the alignment. For the assignment of a consensus symbol 80% of the residues of a column had to belong to the following amino acid classes: negative (−/DE) coloured in yellow, hydroxylic (*/ST) in brown, aliphatic (a/ILV) in dark blue, positive (+/HKR) in red, tiny (t/AGS) in green, aromatic (a/FYWH) in purple, polar (p/CDEHKNQRST) in light orange, hydrophobic (h/ACFGHILMTVWY) in light blue.

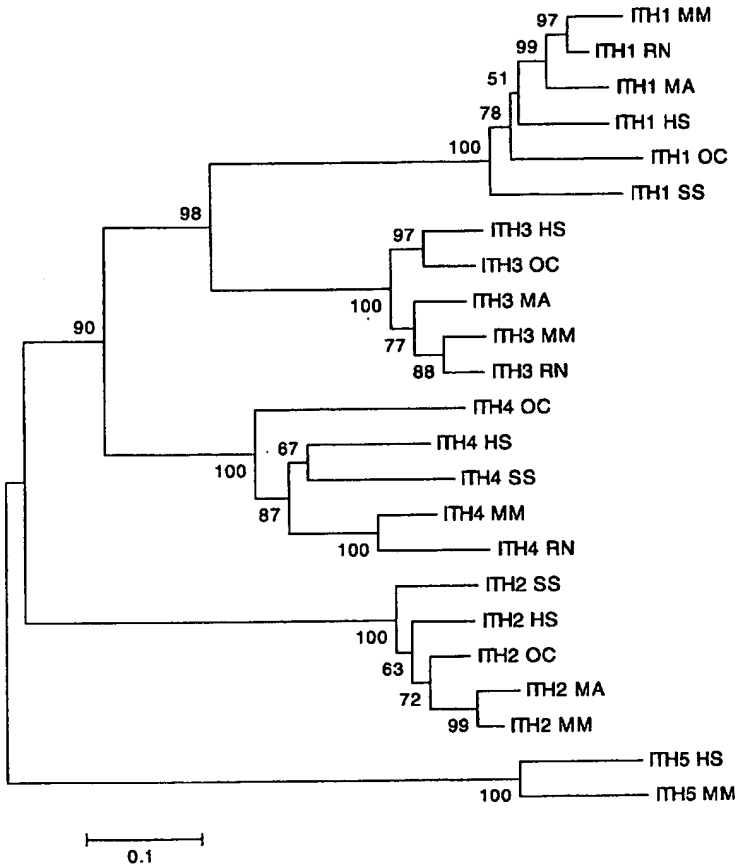
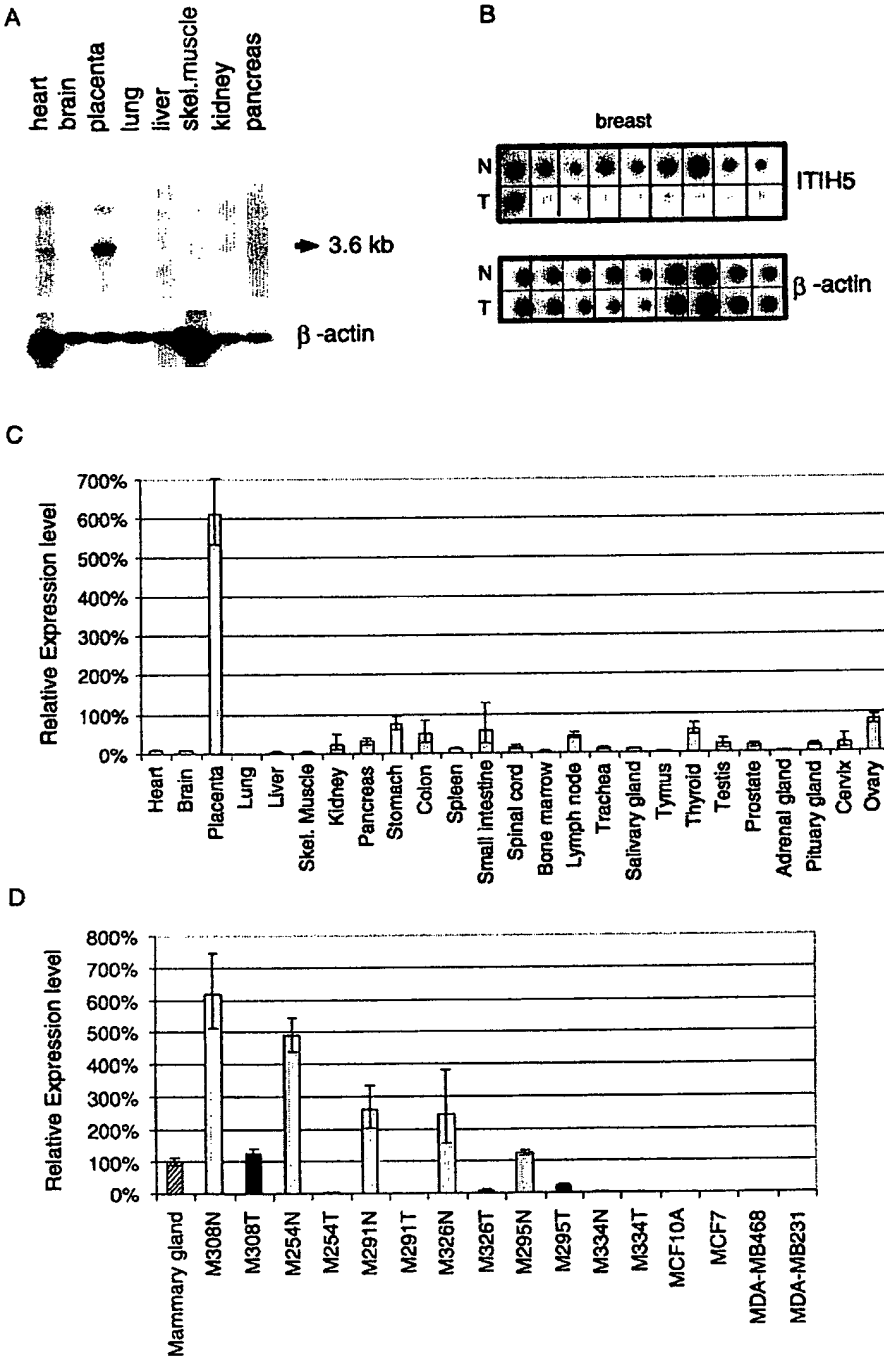


Fig. 3. Position of ITIH5 within the mammalian ITIH superfamily. Phylogenetic tree of known ITIH proteins from human (HS), pig (SS), rabbit (OC), hamster (MA), rat (RN) and mouse (MM) is shown. The tree was built using the neighbour-joining algorithm based on poisson-corrected pairwise distances [11]. The numbers at each node indicate the bootstrap support of the node. The length of a branch is proportional to the evolutionary distance between two taxa/nodes. The scale bar depicts a distance of 0.1 as estimated by a Poisson model of substitutions based on amino acid data as implemented in the MEGA2 program [12].

observed in mammary gland and ovary. Expression was barely detectable in the other tissues examined (Fig. 4C). The fact that *ITIH5* expression was strongest in placenta and some female reproductive tissues is in agreement with published data concerning expression of other ITIH family members in placenta [17] and ovary [18]. So far expression of *ITIH* family members in mammary gland has not been described.

Fig. 4. Expression of *ITIH5* in human normal tissues (A and C), breast tumors (B and D) and breast cell lines (D). Hybridization of poly(A)$^+$ RNA from normal human tissues (multiple tissue Northern blot) and cDNAs from matching breast tumor/normal samples (human matched tumor/normal expression array) were performed with α-^{32}P-labeled fragment of the 3′ *ITIH5* cDNA according to the manufacturer's instructions (Clontech, Palo Alto, USA). Note control hybridization with β-actin (in A and B). Real-time PCR of *ITIH5* was carried out on normal tissues (C) and matching breast normal (grey columns) and tumor (black columns) pairs as well as breast cell lines (D) using the Gen Amp® 5700 sequence detection system (PE Applied Biosystems, Weiterstadt, Germany). Mammary gland cDNA from Clontech was used as reference (100% relative expression; hatched columns in C and D).


A
heart
brain
placenta
lung
liver
skel.muscle
kidney
pancreas
3.6 kb
β -actin
B
breast
N
T
ITIH5
N
T
β -actin
C
Relative Expression level
700%
600%
500%
400%
300%
200%
100%
0%
Heart
Brain
Placenta
Lung
Liver
Skel. Muscle
Kidney
Pancreas
Stomach
Colon
Spleen
Small intestine
Spinal cord
Bone marrow
Lymph node
Trachea
Salivary gland
Tymus
Thyroid
Testis
Prostate
Adrenal gland
Pituary gland
Cervix
Ovary
Uterus
Mammary gland
D
Relative Expression level
800%
700%
600%
500%
400%
300%
200%
100%
0%
Mammary gland
M308N
M308T
M254N
M254T
M291N
M291T
M326N
M326T
M295N
M295T
M334N
M334T
MCF10A
MCF7
MDA-MB468
MDA-MB231

To investigate *ITIH5* expression in breast tumorigenesis we examined breast tumor samples in comparison to normal breast samples of the same patient by two independent techniques. Using dot blot hybridization (Fig. 4B) on Tumor/Normal arrays (Clontech, Palo Alto, USA) we found a significant downregulation of *ITIH5* in breast tumors, i.e. eight of nine analyzed normal/tumor pairs exhibited a more than two-fold downregulation of *ITIH5* expression in the tumor tissue. This observation was confirmed by real-time PCR (Fig. 4D), i.e. all five analyzed normal/tumor pairs exhibited strongly reduced *ITIH5* expression in the tumor. Furthermore, *ITIH5* was undetectable (C_T values >38) in all cell lines examined even those that are considered as benign epithelial cells like MCF10A (examples shown in Fig. 4D). To characterize the cellular expression pattern of *ITIH5* in the mammary gland, we performed radioactive RNA in situ hybridization on breast tumor/normal tissue samples (Fig. 5). A clear expression of *ITIH5* mRNA was detected in the epithelial cells of the normal mammary gland (in 18 of 21 samples analyzed; example shown in Fig. 5A). Expression was detectable in lobules and ducts but not in the stromal cells. This expression level was clearly above the level of background staining detected with the sense probe (Fig. 5B and D). *ITIH5* mRNA was not detectable or only very weakly expressed in invasive ductal carcinomas (in 16 of 20 samples analyzed; example given in Fig. 5C). This observation could argue for a possible role of ITIH5 in breast tumorigenesis. Indeed, the ITIH family proteins are known to play a role in ECM stabilization [18,19]. It has been shown recently [9] that overexpression of *ITIH1* and *-3* causes a reduction in metastasis formation in lung cancer mouse models. Both genes also mediate an increase of cell attachment in vitro. Thus, it is conceivable that the loss of *ITIH5* might have a similar function in tumorigenesis and facilitate the progression to a more invasive tumor phenotype.

In summary, we have characterized a novel heavy chain member of the *ITIH* gene family, *ITIH5*. The association of *ITIH5* to this gene family is based on protein alignments as well as the existence of characteristic domains and a conserved cleavage site. We show that *ITIH5* expression is lost during

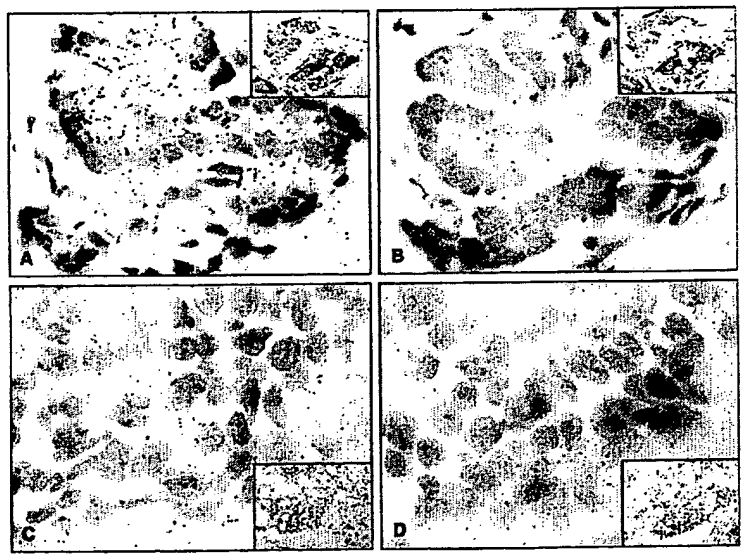


Fig. 5. Downregulation of *ITIH5* in breast cancer. In situ hybridization pattern of *ITIH5* mRNA on normal (A and B) and tumor breast tissue (C and D) (magnification 630 ×). A and C: hybridization with antisense probe; B and D: hybridization with sense probe (negative control). Epithelial cells of normal breast lobuli exhibit clearly detectable *ITIH5* mRNA expression (A). Note that there is no detectable *ITIH5* specific RNA in tumor cells of invasive ductal carcinomas. (C).

breast tumorigenesis. Functional assays to assess a potential role of ITIH5 protein in tissue architecture and tumorgenesis are currently performed in our laboratory.

Acknowledgements

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crease. Many of the responses of the liver to changing metabolic demands are regulated by adrenocortical steroid hormones that affect the synthesis as well as the stability of specific mRNAs in liver cells (Figure 11-4), as we discuss later in this chapter.

The liver also responds to the presence of heavy metals and other toxic substances, which are transported from the gut to the liver via the portal vein. Detoxification of many such noxious chemicals by hepatocytes, the main cell type in the liver, requires special proteins that can metabolize or bind to these compounds. For example, the presence of cadmium or other heavy metals causes the liver to increase production of *metallothioneins,* two small proteins that bind heavy-metal ions and thus provide protection against their toxic effects. Other toxic substances (e.g., phenobarbital, codeine, morphine, and carcinogens such as methylcholanthrene) are detoxified by oxidation. When such substances reach the liver, the synthesis of a group of oxidative enzymes termed the cytochrome P-450s (or simply P-450s), which are present in hepatocytes, increase by a factor of 100 or more. The many individual genes which encode P-450 enzymes respond to different toxic substances, or *xenobiotics.* The molecule or molecules which recognize many of these toxic substances and which thus constitute the active signal for gene control during these metabolic responses in the liver cell are not yet known. However, activation of P-450 genes by cyclic hydrocarbons may resemble the induction of genes by steroids because the liver contains specific proteins that bind toxic substances (e.g., a dioxin derivative) and then enter the nucleus. As we will discuss later, steroids regulate transcription by binding to cytoplasmic DNA-binding proteins that move to the nucleus.

Experimental Demonstration of Transcriptional Control

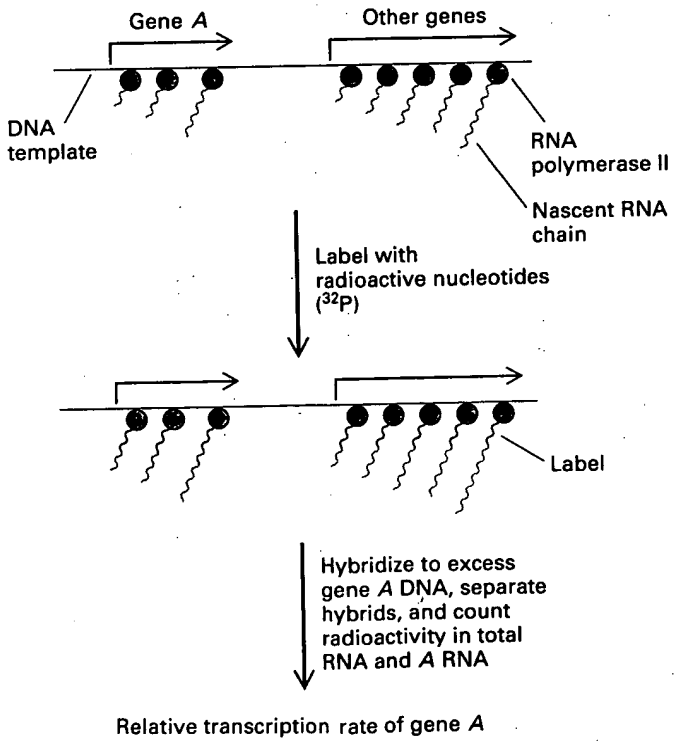
As illustrated in Figure 11-2, gene control can occur at four levels: (1) transcription (either initiation or termination), (2) nuclear processing of primary transcripts, (3) cytoplasmic stabilization or destabilization of RNAs, and (4) mRNA translation. Enough examples have been analyzed to show that gene control at each of these levels occurs in eukaryotes, although not every gene is—or can be—controlled at all four levels.

Regulation of transcriptional initiation is by far the most widespread form of gene control in eukaryotes, as it also is in prokaryotes. Such control results in the increased or decreased synthesis of primary RNA transcripts in response to some signal, leading to a change in the level of specific mRNAs and their translation products. In this section, we describe how control of transcriptional initiation has been demonstrated in several eukaryotic systems. In the following sections, we discuss various aspects of such control and describe several biological events that depend on transcriptional control. After this detailed discussion of transcription-initiation control, the other levels of gene control are considered.

"Run-On" Transcription Analysis Accurately Measures Transcription Rates

The simplest and most direct method of measuring transcription rates would be to expose cells for a brief time (e.g., 5 min or less) to a labeled RNA precursor and measure the amount of labeled nuclear RNA formed by its hybridization to a cloned DNA. This method has been used for transcription rate measurements with cultured cells. However, the technique is not practical in whole animals because the labeled RNA that can be obtained is insufficient.

Even with cultured cells it is often easier to use a second method of labeling RNA. In this method, called "run-on" or nascent-chain analysis, nuclei are isolated from cells and allowed to incorporate ^{32}P from labeled nucleoside triphosphates directly into nascent (growing) RNA chains to produce highly labeled RNA preprations (Figure 11-5).



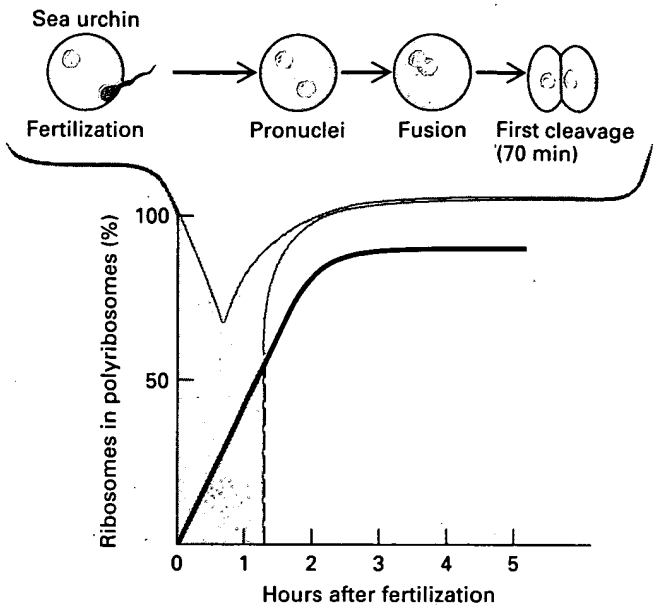
▲ **Figure 11-5** Nascent-chain or "run-on" assay for transcription rate of a gene. Labeled RNA is prepared in isolated nuclei by allowing extension of already initiated RNA chains. The average polymerase only moves a few hundred nucleotides and very little new initiation occurs. By hybridizing the labeled RNA to the cloned DNA from a specific gene (*A* in this case), the fraction of total RNA copied from that DNA can be measured. [See J. Weber, W. Jelinek, and J. E. Darnell, 1977, *Cell* 10:611.]

tions. The intracellular factors (protein?) that recognize these stem-loops and regulate this iron-dependent mRNA destruction are being studied at present.

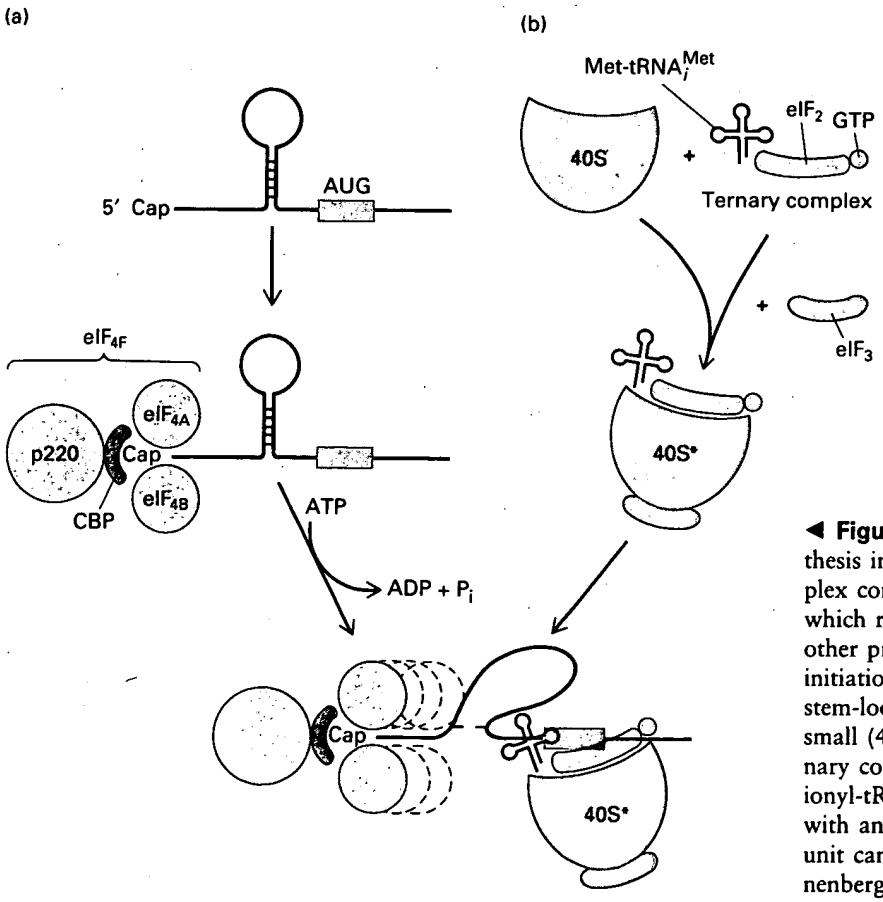
Overall Rate of mRNA Translation Can Be Controlled

Translational control of specific proteins has been proved in only a few cases. In contrast, well-known mechanisms of translational control for all mRNAs operate in all cells to change the *average* rate of protein synthesis. For example, when cultured mammalian or insect cells are heated above 40°C, most translation initiation is suppressed, but the frequency of initiation of translation of heat-shock mRNA actually rises. As we mentioned earlier, an increase in the transcription of the heat-shock genes accompanies the increased translation of heat-shock mRNAs. Mammalian cells adapt to the new elevated temperature (as long as the temperature is not higher than about 42°C) and resume normal protein synthesis and growth within 2 h after a heat shock.

A similar general inhibition of protein synthesis occurs when cells enter mitosis, which causes a fall in the rate of protein synthesis to 30 percent of normal. This decline in protein synthesis is accompanied by a decrease in the size



▲ **Figure 11-50** Translational control of protein synthesis in the early cleavage stages of invertebrate embryogenesis. The percentage of ribosomes in polyribosomes (red curve), which is an index of translation, rises in fertilized sea urchin eggs using preexisting maternal mRNA. [See B. Brandhorst, 1976, *Devel. Biol.* 52:310.]



◀ **Figure 11-51** Model of initiation of protein synthesis in eukaryotes. (a) The cap-binding (CB) complex consists of a 24-kDa cap-binding protein (CBP), which recognizes the 5′ cap in mRNA, and several other proteins, collectively referred to as eukaryotic initiation factor 4F (eIF_{4F}), that probably unwind the stem-loops near the 5′ end. (b) Activation of the small (40S) ribosomal subunit requires eIF_3 and a ternary complex consisting of eIF_2, GTP, and methionyl-$tRNA_i^{Met}$. After the CB complex has associated with an mRNA molecule, an activated ribosomal subunit can locate the AUG start codon. [See N. Sonnenberg, 1987, *Adv. Virus Res.* 33:172.]

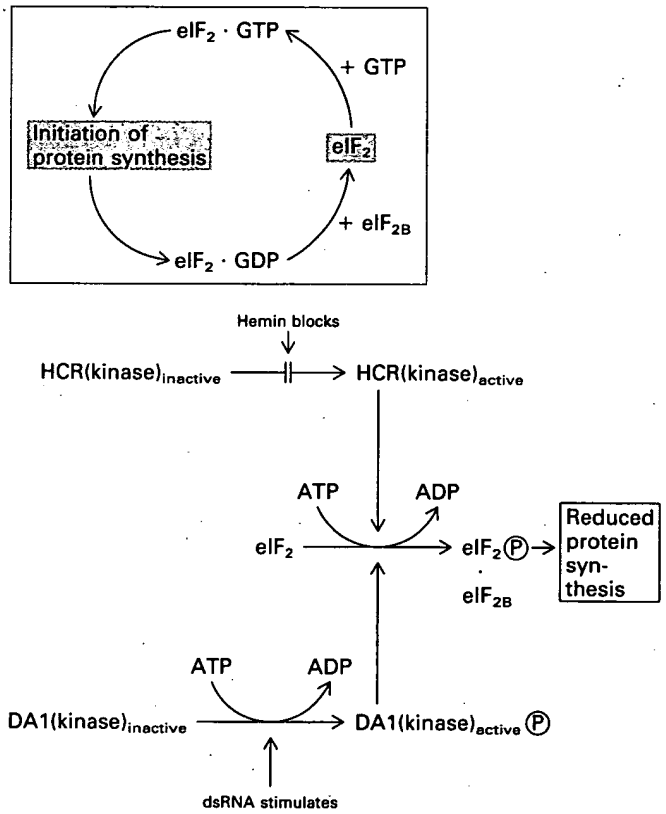
of polyribosomes; that is, fewer ribosomes are attached to each mRNA, indicative of decreased initiation with a normal rate of polypeptide elongation and termination. There is also a great decrease in mRNA synthesis during mitosis, but the decline in the number of mRNA molecules is not sufficient to account for the decrease in protein synthesis; translational control is clearly the basis for the decrease.

Protein synthesis in unfertilized invertebrate eggs is very slow, but after fertilization it increases dramatically in the absence of any formation of new mRNA. Thus protein synthesis during the early stages of embryogenesis represents the delayed use of stored "maternal" mRNAs whose translation is inhibited in the unfertilized egg (Figure 11-50).

These examples indicate that mechanisms are available in cells for regulating the overall rate of mRNA translation under a number of different conditions. To understand how the efficiency of translation might be regulated, we need to consider the process of translational initiation. As currently envisioned, the initiation of protein synthesis requires recognition by a complex of proteins of the cap structure at the 5′ end of an mRNA, followed by unwinding of any stem-loop structure near the 5′ end. This allows recognition of the AUG start codon by a 40S-ribosomal initiation complex. The components that recognize and bind to the cap (Figure 11-51) include a 24-kDa protein, which specifically recognizes the cap structure, and at least three other proteins, which together are referred to as eukaryotic initiation factor 4F eIF_{4F}. The ribosomal initiation complex consists of the 40S subunit, eIF_3, and a "ternary complex" consisting of eIF_2, GTP, and initiator methionyl-$tRNA^{Met}$ (Met-$tRNA_1^{Met}$).

Much evidence indicates that the greater the secondary structure at the 5′ end of an mRNA, the more difficult it is to start translation and the greater the requirement for eIF_{4F} activity. For example, oligonucleotides complementary to the first 15 bases of mRNAs greatly inhibit initial ribosome binding, and mRNAs with little or no secondary structure at their 5′ end associate well with ribosomes without assistance of eIF_{4F} or at much lower than normal concentrations. The heat-shock mRNAs, the only mRNAs that are translated efficiently in heat-shocked cells, have a very A-rich 26-nucleotide stretch before their initiation codon; such A-rich stretches do not form extensive secondary structures. These results suggest that translation control might be effected by substances that increase or decrease the secondary structure at the 5′ ends of mRNAs, but well-documented instances of regulation using these principles have yet to be described.

A second means of regulating the efficiency of translational initiation is modification of an initiation factor. One example of this mechanism is phosphorylation of initiation factors by protein kinases, which add phosphates to serine, threonine, and tyrosine residues. The biochemistry of translational initiation in mammalian cells has been studied most extensively in reticulocytes because a number of the components of their translational machinery have been purified. When eIF_2 is phosphorylated, it is inactive (Figure 11-52). In reticulocyte extracts, the trigger for phosphorylation by a protein kinase can be either the absence of heme (a component of hemoglobin) or the presence of a segment of double-stranded RNA (e.g., during RNA virus infection). Since hemoglobin contains both heme and the globin chains, a balance of the two is assured by this translational control; a decrease in heme leads to a decrease in globin mRNA translation. It is likely that all cells possess these protein kinases and that many factors other than heme and double-stranded RNA may affect their activity.



▲ **Figure 11-52** Translation control by phosphorylation of eukaryotic initiation factor 2 (eIF_2). As shown in Figure 11-51, eIF_2 is required to initiate protein synthesis. Two different protein kinases, HCR kinase and DA1 kinase, can phosphorylate eIF_2. Phosphorylated eIF_2 gets trapped by binding tightly to eIF_{2B}, a protein that normally regenerates free eIF_2. Inactive HCR kinase is activated by the absence of hemin, which is a derivative of heme. Inactive DA1 kinase is activated by double-stranded RNA (dsRNA). Thus either the absence of heme (conceivably due to iron starvation) or the presence of double-stranded RNA (e.g., during RNA virus infection) leads to phosphorylation of eIF_2 and decreased initiation of protein synthesis. [See P. J. Farrell et al., 1977, *Cell* 11:187; and N. Sonenberg, 1987, *Adv. Virus Res.* 33:172.]

Differential Translational Control of Specific mRNAs Is Not Common in Eukaryotes

In the previous section, we described several cases of repression or stimulation of the overall translation rate. To date, relatively few examples of differential translation of specific mRNAs are well studied. One example is the surf clam, *Spisula solidissima,* in which synthesis of some proteins decreases after fertilization, while the synthesis of other proteins rises sharply. However, if the total mRNA extracted from both fertilized and unfertilized clam eggs is translated in vitro, the resulting protein patterns are very nearly identical. Thus the same mRNAs are present in clam eggs before and after fertilization but their translation is differentially regulated. The untranslated mRNAs in both fertilized and unfertilized eggs are not associated with polyribosomes, indicating that initiation of translation is the regulated step of synthesis.

In Chapter 7 we noted that translational control operates for a number of bacterial ribosomal proteins (those proteins associated with ribosomal RNA in ribosomes). Specific control of translation of mRNAs encoding ribosomal proteins also occurs in mammalian cells. When growth is suspended by placing cultured cells in a medium lacking all growth factors, the mRNAs for ribosomal proteins are not associated with polyribosomes and are no longer translated. When serum is returned to the medium, these mRNAs again become engaged by the translation initiation apparatus and are translated.

Another example of specific translational control involves the iron-binding protein ferredoxin, which is synthesized faster when cells are placed in a medium of high iron content. A protein has been purified that specifically binds to a stem-loop region in the 5′ untranslated region of ferredoxin mRNA and prevents translation. This translation-inhibitor protein does not inhibit translation of ferredoxin mRNA in iron-rich circumstances but does when a chelator is present. The stem-loop region in the ferredoxin mRNA is about 12 base pairs long and contains a CAGUG sequence, the same sequence that is present in the 3′ untranslated regions of the transferrin-receptor mRNA mentioned earlier. Whether the same protein mediates destruction of transferrin-receptor mRNA and inhibit ferredoxin mRNA translation at high iron concentrations is not yet known.

Summary

The primary function of gene control in single-cell organisms is to respond to alterations in their environment, particularly changes in the available nutrients. Because cells in multicellular organisms are shielded from acute environmental changes, they have less need for this type of gene control, and their ability to respond to nutritional deprivation and other environmental stresses is limited. The primary function of gene control in multicellular organisms is to direct developmental pathways that result in many different types of cells, each characterized by a particular protein composition that endows cells with specific functions. The most common gene-control signals in animals are hormones (and hormonelike substances), which include small molecules that can diffuse into cells, circulating polypeptides that bind to cell-surface receptors and cell-cell and cell-matrix contacts.

Gene regulation occurs at several levels in eukaryotes, the primary and most frequent being control of transcriptional initiation. Although several biological systems using regulated transcriptional termination have been identified, this mechanism is not common in eukaryotes. Transcriptional initiation depends on many different nuclear proteins that assist RNA polymerase II in starting transcription. The genes encoding dozens of these factors have been cloned and sequenced, and the amino acid sequences of their protein products have been determined. At present several major types of regulatory proteins are recognized based on their characteristic structural motifs: helix-turn-helix (including homeobox) proteins; zinc-finger proteins; and two groups of dimeric amphipathic helical proteins, the leucine-zipper and helix-loop-helix proteins. There are also a number of regulatory proteins that do not fall into these categories.

Most of the eukaryotic transcription factors that have been identified exert a positive effect, although negative-acting proteins are known to occur in some systems. In general, to exert their effect these proteins must bind to specific DNA sequences, some of which may be quite distant (either upstream or downstream) from the start site of a gene. Some regulatory proteins appear to act as modifiers and exert their effect by protein-protein interactions with other factors that bind to DNA. Cell-specific gene expression thus depends on the cell-specific differential activity of regulatory proteins, which can be controlled by their cell-specific (or cell-limited) synthesis and by cell-specific interconversion between active and inactive forms. In eukaryotes, transcription is affected by chromatin structure, and transcriptionally active regions of DNA are characterized by a "looser" chromatin structure and undermethylation of certain residues. Networks of interdependent regulatory factors are under study in several situations. The genes responsible for two cell states in yeast (a and α) and how these genes are coordinately regulated is fairly well understood. Also the gene regulation underlying switching from a to α is becoming better understood. During early *Drosophila* embryogenesis there is a sequential cascade of transcription factors responsible for directing early larval development. Finally, studies on mammalian regulatory factors that participate in organ-specific expression have been identified and are under study.

Differential RNA processing is the second major gene-control mechanism, especially in animals. The cell-specific production of different proteins from complex primary transcripts has been shown to depend on differential selection of poly A sites and/or splice sites. Although the molecular basis for differential processing is not known, cell-specific variations in the components of the processing machinery (i.e., snRNPs) have been demonstrated in a few cases.

Short-lived mRNAs are characterized by the absence of poly A tails (e.g., histone mRNA) or by the presence of $A(U)_nA$ sequences in their 3′ untranslated end. Differential stabilization or destabiliation of specific mRNAs by hormones, translation products, and ligands has been demonstrated, and this mechanism of gene control is fairly widespread in eukaryotes. Rapid mRNA turnover usually does not occur if translation is blocked, suggesting that the nucleases responsible for mRNA degradation are associated with ribosomes.

Initiation of translation depends on the interaction of several proteins with the 5′ end of an mRNA molecule, which leads to loosening of the mRNA secondary structure in this region; as a result, an activated small ribosomal subunit can locate the start codon. Several mechanisms for regulating the rate of translational initiation can be envisioned, and the overall rate of mRNA translation is known to vary under certain conditions. However, only a few cases of differential translation of specific mRNAs have been demonstrated so far. Thus, the importance of translation control to differential gene expression in eukaryotes is still less clear than other mechanisms.

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Aligning...
Sequences (1:2) Aligned. Score:  99
Start of Multiple Alignment
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                                   **************************************************

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g30314037_ITIH5_human           IVFLTDGKPTVGETHTLKILNNTREAARGQVCIFTIGIGNDVDFRLLEKL
                                **************************************************

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                                **************************************************

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                                **************************************************

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